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MATERIALS AND METHODS

Animals

One heterozygous male TgN(GFPU)5Nagy mouse and one wild-type strain female mouse were purchased from the Jackson Laboratory (Bar Harbor, ME) as a breeding pair. TgN(GFPU)5Nagy mice created by germline transmission of a 129 ES line (H-2^b haplotype) were maintained by crossing with ICR (outbred) mice.[34] Male TgN(GFPU)5Nagy offspring were identified by analysis of tail biopsies using a Zeiss fluorescent microscope and used as SC donors. Male SCID (Taconic Farms) and Balb/c (H-2^d haplotype; University of Alberta, Health and Laboratory Animal Services) mice, aged 6-8 weeks, were used as recipients.

Sertoli Cell Isolation and characterization

SCs were isolated using a technique similar to that previously described for rat SCs.[9] Briefly, testicles from adult male TgN(GFPU)5Nagy transgenic mice were surgically removed and placed in a 50 ml conical tube containing cold (4°C) Hank's balanced salt solution (HBSS) supplemented with 0.25 % (w/v) fraction V bovine serum albumin (BSA; Sigma Chemical Co., St. Louis, MO). The testes were cut into 1-mm fragments with scissors, digested for 6 min at 37°C with collagenase Type V (2.5 mg/ml; Sigma) and then washed 3 times with HBSS. The tissue was resuspended in calcium-free medium supplemented with 1 mM EGTA and further digested with trypsin (25 μg/ml; Boehringer Mannheim, Laval, Canada) and DNase (4 μg/ml, Boehringer) for 10 min at 37°C. The digest was passed through a 500-μm nylon mesh, washed with HBSS and cultured in non-treated petri dishes (10 cm diameter) containing Ham's F10 media supplemented with 10 mmol/l D-glucose, 2 mmol/l L-glutamine, 50 μmol/l

isobutylmethylxanthine, 0.5% BSA, 10 mmol/l nicotinamide, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% fetal calf serum. Cells were incubated for 48 hours at 37°C to allow the formation of SC aggregates (100 to 300 μm diameter) before transplantation under the kidney capsule.

Sertoli Cell Transplantation

To determine the number of SCs isolated, three representative aliquots of the cell suspension were measured for total cellular DNA content using a Hoefer DyNa Quant 200 fluorometric assay (Amersham Pharmacia Biotech, San Francisco, CA). Aliquots were washed with citrate buffer (150 mmol/l NaCl, 15 mmol/l citrate, 3 mmol/l EDTA, pH 7.4), resuspended in TNE buffer (10 mM tris, 0.2 mM NaCl, 1 mM EDTA, pH 7.4) and sonicated. Aliquots of 10 μl were assayed in triplicate by diluting them in 2 ml of assay solution (0.1 μg/ml Hoechst 33258 in 1 X TNE) and measuring fluorescence (365 nm excitation / 460 nm emission). A six-point (0-500 ng/ml) DNA standard curve was generated using calf thymus DNA. For transplantation, aliquots consisting of 12.68 $\pm$ 5.38 million SCs as aggregates (6.6 pg DNA/cell) were aspirated into polyethylene tubing (PE-50), pelleted by centrifugation, and gently placed under the left renal subcapsular space of Halothane-anesthetized SCID (n=6) and BALB/c (n=10) mice.[24]

Diabetic mice

Balb/c mice were rendered diabetic by intraperitoneal injection of streptozotocin (Streptozotocin, Sigma, St. Louis, MO) at a dose of 275 mg/kg body weight. Diabetes was confirmed by the presence of hyperglycemia and only those mice with non-fasting blood glucose levels exceeding 20 mM were used as recipients. Islets were isolated from TgN(GFPU)5Nagy

mice by collagenase (Sigma, Type V) digestion, dextran gradient purification and subsequently cultured 24 hours prior to transplantation of 500 TgN(GFPU)5Nagy mouse islets underneath the renal capsule of diabetic Balb/c mice.[9] After transplantation, non-fasting blood glucose levels were measured twice a week and graft rejection was defined as two successive blood glucose levels >14 mM.

Graft Characterization

Nephrectomies were performed at 30 and 60 days post-transplant and the graft-bearing kidneys were examined immediately using blue-light fluorescent microscopy for GFP expressing cells underneath the kidney capsule. Kidneys were then immersed in Z-fix and embedded in paraffin. After deparaffinization, and rehydration, sections were heated for 15 min in 0.01M sodium citrate buffer (pH 6.0), in a microwave at full power.[9,24] Sections were incubated with 10% hydrogen peroxide to quench endogenous peroxidases, blocked with 1% BSA, and incubated with goat polyclonal anti-GATA-4 (1:50; Santa Cruz Biotechnology, Santa Cruz, CA) primary antibody for 30 minutes, followed by biotinylated horse anti-goat secondary antibody for 20 minutes and diaminobenzamide as the chromagen using an ABC kit (Vector Laboratories, Burlingame, CA). Sections were then incubated with mouse monoclonal anti-GFP (1:100; Chemicon International, Inc., Temecula, CA) primary antibody for 30 minutes, followed by Cy3-conjugated goat anti-mouse secondary antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) for 20 min.

RESULTS

Characterization of Sertoli cells from TgN(GFPU)5Nagy mice

To verify the presence of GFP in SCs isolated from TgN(GFPU)5Nagy transgenic mice, testicular sections from TgN(GFPU)5Nagy and wild-type mice were immunostained for GFP (Fig.1 A, B). GFP positive SCs were present in the TgN(GFPU)5Nagy but not the wild-type testicular sections. To further verify the expression of GFP by SCs, seminiferous tubules and SCs were isolated from TgN(GFPU)5Nagy mouse testes and examined under blue-light fluorescence. An aliquot containing seminiferous tubules was collected during the isolation procedure after collagenase digestion. These tubules contained many GFP expressing cells (Fig. 1 C). At the time of transplantation, SCs that had been cultured for two days to form aggregates were analyzed and also contained GFP expressing cells (Fig. 1 D, E). Since the seminiferous tubules and aggregates contain other cells including germ cells and peritubular myoid cells, isolated cells were also cultured on chamber slides as a monolayer. SCs cultured in a monolayer can be identified by their characteristic morphology. These SCs expressed GFP (Fig. 1 F). Nonetheless, it should be noted that not all of the cells from the TgN(GFPU)5Nagy mouse testes expressed GFP (Fig. 1 D). SCs isolated from Balb/c mice were not fluorescent (data not shown).

Transplantation of TgN(GFPU)5Nagy Sertoli cell aggregates to SCID mice

To asses whether SCs that produce GFP would be able to survive transplantation and continue to express the transgene, SC aggregates expressing GFP were transplanted under the left kidney capsule of immunocompromised SCID mice. Nephrectomies were performed after 30 and 60 days and kidneys were analyzed immediately with blue light illumination for the

presence of GFP positive cells under the kidney capsule. Many brightly fluorescent GFP positive cells were detected in each of the grafts (Fig. 2 A, B). Negative controls consisting of the kidney from a SCID mouse did not fluoresce (Fig. 2 C).

To be certain the GFP positive cells detected in the grafts were SCs, sections from grafts 30 and 60 days post-transplant were double immunostained for GFP and GATA-4 (a marker for SCs).[24] GFP positive SCs were present in 100% of the grafts at both 30 and 60 days post-transplant (Fig.3). However, not all of the SCs expressed GFP. This is consistent with the variable expression of GFP in the SCs from the TgN(GFPU)5Nagy mouse testes.

Transplantation of TgN(GFPU)5Nagy mouse islets to Balb/c mice

Prior to testing the survival of GFP expressing SCs as allografts, it was first necessary to confirm that nonimmunoprivileged cells from the genetically undefined TgN(GFPU)5Nagy mice would be immunologically rejected by H-2^d Balb/c mice. Islets isolated from TgN(GFPU)5Nagy mice were transplanted to the renal subcapsular space of diabetic Balb/c mice and all animals rejected these islets within 17 days, mean graft survival 14.75 ± 1.5 days + SE (Fig. 4).

Transplantation of TgN(GFPU)5Nagy Sertoli cell aggregates to Balb/c mice.

To determine whether GFP expressing SCs can survive as allografts and continue to produce the transgene, SC aggregates expressing GFP were transplanted under the left kidney capsule of Balb/c mice. Nephrectomies were performed after 30 and 60 days and kidneys were analyzed immediately under blue light illumination for the presence of GFP positive cells under the kidney capsule. GFP positive cells were found in all the grafts at both 30 and 60 days post-

transplant (Fig. 5 A-D). In fact, some of the grafts (4 of 10) contained fluorescent tubules (Fig. 5 B, D). The kidney from a Balb/c mouse was used as a negative control and did not fluoresce (Fig. 5 E).

To confirm that the GFP positive cells detected in the grafts were SCs, sections from grafts 30 and 60 days post-transplant were immunostained for GFP and GATA-4. GFP positive SCs were detected in grafts from Balb/c mice at both 30 and 60 days post-transplant (Fig. 6). Seven of the ten grafts were positive for GATA-4 while 6 of the 10 grafts were positive for GFP. When sections were double stained for GFP and GATA-4, GFP positive SCs were present in 50% of the grafts (Fig.6). This data supports the hypothesis that genetically engineered SCs can survive as allografts and express genes of interest.